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(54) Title: XANTHONE ANALOGS FOR THE TREATMENT OF INFECTIOUS DISEASES			
(57) Abstract Therapeutic compositions for the treatment of infectious diseases are disclosed. These compositions comprise xanthenes and xanthone derivatives, such as 2,3,4,5,6-pentahydroxyxanthone. Also disclosed are methods for the treatment of infectious diseases using such compounds.			

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XANTHONE ANALOGS FOR THE TREATMENT
OF INFECTIOUS DISEASES

TECHNICAL FIELD

5 This invention pertains to therapeutic compositions for the treatment of infectious diseases.

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10 This invention was made with partial support from the United States Government to Drs. Michael K. Riscoe and David J. Hinrichs through the Veterans Affairs Merit Review System. The U.S. Government may have certain rights to this invention.

15 BACKGROUND OF THE INVENTION

 Protozoan parasites cause diseases such as malaria, trypanosomiasis, Chagas' disease, leishmaniasis, giardiasis, and amoebiasis. These and other protozoan parasite diseases have historically occurred in tropical and sub-tropical areas where they cause widespread damage to human populations. Although they receive little attention in the Western world, protozoan diseases affect more people worldwide than diseases brought on by any other biological cause (Heyneman, 1988).

25 Today, malaria remains the most destructive single infectious disease in the developing world. It is responsible for more human energy loss, more debilitation, more loss of work capacity, and more economic damage than any other human ailment facing the world today (Heyneman, 1988). The World Health Organization estimates that 1 to 2 million deaths are caused by malaria each year in Africa alone; most of these are children under the age of five (World Health Organization, 1991). In addition, over 300 million people worldwide are believed to be chronically infected, and each year nearly one third of these

individuals will suffer acute manifestations of the disease.

Today, the pathologic capacity of protozoa is being increasingly demonstrated in the Western world among the victims of AIDS (Acquired Immunodeficiency Syndrome). AIDS depletes the immune system of affected individuals; this allows opportunistic agents which would be defeated by an active immune system to infect AIDS patients. Several protozoa have emerged as important opportunistic infections in AIDS patients including *Cryptosporidium parvum*, *Entamoeba histolytica*, *Giardia lamblia*, *Pneumocystis carinii* (which may be a fungal or protozoal pathogen), and *Toxoplasmosis gondii*.

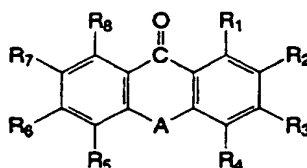
Despite the prevalence and significance of protozoan infections, therapy for these diseases is generally poor or in need of improvement. Many chemotherapeutic agents used to treat protozoan infections are non-specific cytotoxins that are highly toxic and cause severe side effects in patients. However, these drugs are used because there are no better alternatives. For example, giardiasis and amoebiasis are treated using metronidazole (a nitroimidazole), but the use of this drug is clouded by its mutagenic potential (Campbell, 1986) and its adverse interaction with alcohol. For trypanosomiasis and leishmaniasis standard therapies (suramin, melarsoprol, and pentavalent antimonials) are dangerously toxic, occasionally fatal, and often ineffective (Mebrahtu, 1989; Grogl et al., 1992). Other drugs are becoming ineffective due to emerging resistance. In the case of malaria, effective therapy has previously been provided by chloroquine but its efficacy is now threatened by the rapid emergence of drug resistant strains of *Plasmodium falciparum*, the causative agent for the most severe, often fatal, form of the disease (Cowman, 1990). Other protozoal infections such as cryptosporidiosis or Chagas' disease have no proven curative agent.

The present invention is directed to new antiparasitic agents and methods of using known compounds as anti-parasitic agents. The invention is also directed to compounds with broad-spectrum anti-microbial activity.

SUMMARY OF THE INVENTION

As a result of studies aimed at developing new anti-parasitic agents, the present inventors have discovered that xanthenes and a wide range of xanthone derivatives and structurally related compounds, as shown in formula X1 below, have potent anti-parasitic activity. The compounds have broad-spectrum anti-microbial activity, including anti-fungal activity against *Candida albicans* and *Apergillus fumigatus*.

Formula X1:



wherein:

A is oxygen, substituted antimony (stibium), sulfur or N-R' wherein R' is H, OH, alkyl, haloalkyl, aryl or haloaryl; and

R₁-R₈ are independently selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, alkene, substituted alkyl (such as alkylamine, alkylthio and haloalkyl), amino, ester, ether and nitro groups and O-linked and C-linked carbohydrates.

Examples of substituted antimony include antimonial oxides and antimony substituted with hydroxy, chlorine, alkyl and aryl groups (e.g. SbCl, SbCl₃, SbOH, Sb(O)(OH)).

In another embodiment, A is oxygen, sulfur or NH, and R₁-R₈ are independently selected from the group

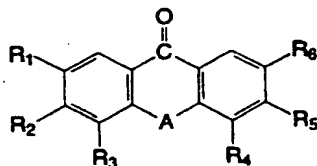
consisting of H, OH, aryl, haloaryl, arylamine, alkyl, alkene substituted alkyl, halogen, amino, ester, ether and nitro groups.

In other embodiments, A is oxygen and R_1 - R_8 are independently selected from the group consisting of H, OH, and acetoxy ($\text{CH}_3\text{C}(\text{O})\text{O}$).

The formula X1 compounds encompass some compounds described for the first time herein.

In other embodiments, the compounds of the present invention include compounds according to formula X2:

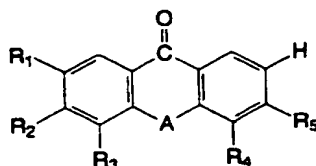
Formula X2:



wherein A is oxygen, sulfur or NH, and R_1 - R_8 are independently selected from the group consisting of H, OH, aryl, arylamine, alkyl, alkene substituted alkyl, halogen, amino, ester, ether and nitro groups. In particular embodiments of the present invention, the term "alkyl" refers to substituents having lower alkyl groups, i.e., C_1 - C_{10} .

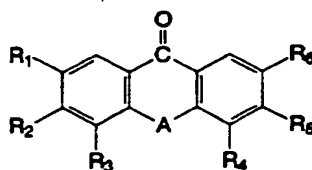
In preferred embodiments of the present invention, the formula X1 compounds are compounds according to the formulae:

Formula X3:



Formula X4:

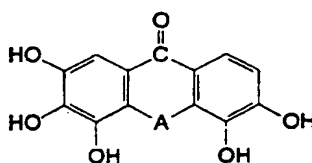
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wherein A is selected from the group consisting of oxygen, sulfur and NH, and R_1 - R_6 are independently selected from the group consisting of H, OH or an ester group, such as OCOCH_3 or $\text{OCO}(\text{CH}_2)_3\text{CH}_3$ or a carbamate ester. In preferred embodiments, the R_2 and R_5 groups are esters. In yet other embodiments, A is oxygen and R_2 - R_5 are all hydroxy; in further preferred embodiments, at least one of R_1 and R_6 are H.

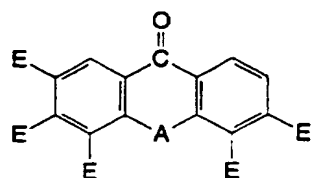
In more preferred embodiments, the formula X1 compounds are compounds having the following formulae:
Formula X5:

20



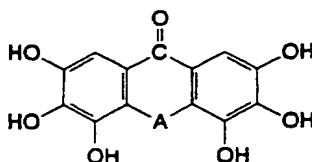
25 Formula X5E:

30



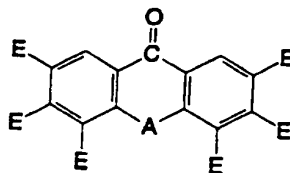
Formula X6:

35



Formula X6E:

5



wherein A is oxygen, sulfur or NH and E is an ester.
 10 Specific examples of such compounds include 2,3,4,5,6-pentahydroxyxanthone; 2,3,4,5,6,-pentaacetoxyxanthone; 2,3,4,5,6,7,-hexahydroxyxanthone; 2,3,4,5,6,7,-hexaacetoxyxanthone; 2,3,4,5,6-pentahydroxyacridone; 2,3,4,5,6,-pentaacetoxiacridone; 2,3,4,5,6,7,-hexahydroxyacridone; 2,3,4,5,6,7,-hexaacetoxiacridone;
 15 2,3,4,5,6-pentahydroxythioxanthone; 2,3,4,5,6,-pentaacetoxythioxanthone; 2,3,4,5,6,7,-hexahydroxythioxanthone; and 2,3,4,5,6,7,-hexaacetoxythioxanthone.

20 In the most preferred embodiments, the formula X1 compounds are 2,3,4,5,6,-pentahydroxyxanthone and esterified forms of this compound, including 2,3,4,5,6-pentaacetoxyxanthone and 2,3,4,5,6,7-hexahydroxyxanthone and esterified forms of this compound, including
 25 2,3,4,5,6,7-hexaacetoxyxanthone.

The present invention thus includes compositions for the treatment of microbial diseases such as malaria, the compositions including a compound according to formula X1. Also included in the present invention is a
 30 method of inhibiting the growth of a microbial pathogen comprising providing a sufficient amount of a formula X1 compound and contacting the microbial pathogen with this compound. Such a method is applicable to inhibit microbial growth *in vivo* and *in vitro*. In one aspect,
 35 the present invention provides a method of treating a patient having a microbial infection, the method comprising administering to the patient a

therapeutically effective amount of a compound according to formula X1.

Another aspect of the present invention is the discovery that certain compounds having the underlying xanthone ring structure depicted in formula X1 bind to, and inhibit the polymerization of, heme. A number of pathogens, including *Plasmodium*, a causative agent of malaria, degrade hemoglobin to obtain amino acids, and in so doing liberate toxic heme (Olliaro and Goldberg, 1995). To avoid the toxic effects of the liberated heme, these pathogens polymerize the heme to form hemozoin. The compounds disclosed herein which are shown to inhibit heme polymerization may thus be used to block heme polymerization and therefore to treat infections caused by these pathogens. It is self-evident that these heme complexing compounds may kill pathogens by preventing these organisms from gaining access to the host's supply of heme iron, or by causing build-up of toxic levels of heme in the organism's vacuole.

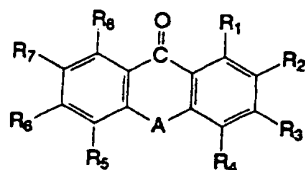
Compounds which are herein disclosed to inhibit heme polymerization may be represented by the structure

$$X - Y - Z$$

wherein X is a group capable of interacting with the iron atom in heme;

Y is a planar aromatic system capable of interacting with the porphyrin ring of heme through overlapping pi-pi orbitals; and

Z represents one or more groups capable of interacting with at least one carboxylate side group of heme. In preferred embodiments, these compounds are formula XH compounds, having a structure:



wherein:

A is oxygen, substituted antimony (stibium), sulfur or N-R' wherein R' is H, OH, alkyl, haloalkyl, preferably lower alkyl or lower haloalkyl wherein

5 "lower" means 10 or fewer carbon atoms, aryl or haloaryl;

R₁-R₃ and R₆-R₈ are independently selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, substituted alkyl (such as alkoxy, alkylamine, alkylthio and haloalkyl), amino, ester and nitro groups
10 and O-linked and C-linked carbohydrates;

at least one (and preferably both) of the R₄ and R₅ substituent pair is selected from the group consisting of amino, substituted amino, alkylamino, substituted
15 alkyl amino, arylamino, amidinium, alkylamidinium, guanidinium, alkylguanidinium, hydroxy, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy groups, COOH, alkyl-COOH, CONH₂ and alkyl-
20 CONH₂; and

the other member of the R₄ and R₅ substituent pair is selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, substituted alkyl (such as alkoxy, alkylamine, alkylthio and haloalkyl), amino,
25 substituted amino, ester and nitro groups, O-linked and C-linked carbohydrates, alkylamino, substituted alkyl amino, arylamino, amidinium, alkylamidinium, guanidinium, alkylguanidinium, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy groups, COOH, alkyl-COOH, CONH₂ and alkyl-
30 CONH₂. In preferred embodiments, both R₄ and R₅ are selected from the group consisting of amino, substituted amino, alkylamino, substituted alkyl amino, arylamino, amidinium, alkylamidinium, guanidinium,
35 alkylguanidinium, hydroxy, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy

groups, and carboxamide derivatives of hydroxy, alkylhydroxy and alkoxyhydroxy groups.

Examples of formula XH compounds include 4,5-dihydroxyxanthone, 2,3,4-trihydroxyxanthone, 3,4,5,6-tetrahydroxyxanthone, 2,3,4,5,6-pentahydroxyxanthone, 1,3,5,6,7-pentahydroxyxanthone, 2,3,4,5,6,7-hexahydroxyxanthone; 4,5-bis-((β -diethylamino)ethoxy)xanthone; and 3,6-dihydroxy-4,5-bis-(piperidinomethyl)xanthone.

The present invention thus also includes compositions for the treatment of diseases such as malaria, which are caused by pathogens that polymerize heme, the compositions including a compound according to formula XH. Also included in the present invention is a method of inhibiting the growth of a such a pathogen comprising providing a sufficient amount of a formula XH compound and contacting the pathogen with this compound. Such a method is applicable to inhibit pathogen growth *in vivo* and *in vitro*. In one aspect, the present invention provides a method of treating a patient having malaria, the method comprising administering to the patient a therapeutically effective amount of a compound according to formula XH.

The invention also contemplates that formula X1 and XH compounds can be administered to patients in a pro-drug form, typically a corresponding substituted benzophenone which will react with oxidant radicals under physiological conditions to produce the active formula X1 or XH compound (Winter et al., 1996).

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 illustrates a proposed mechanism for the formation of 2,3,4,5,6-pentahydroxyxanthone from the metabolic activation of exifone by rufigallol within a red blood cell infected with the *Plasmodium* parasite.

Fig. 2 is a schematic depiction of hemoglobin digestion (with the concomitant release of heme) by the intracellular parasite *Plasmodium falciparum*.

Fig. 3 is a graph showing the inhibition of in vitro heme polymerization by compound X5 (A=O) (2,3,4,5,6-pentahydroxyxanthone). Heme and X5 (A=O) concentrations were 25 μ M. Open diamonds indicate heme alone (control), filled diamonds represent heme and X5 (A=O) together.

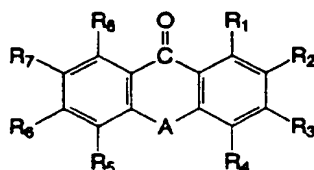
Fig. 4 is a computer simulation of compound X6 (A=O) (2,3,4,5,6,7-hexahydroxyxanthone) complexing with free heme.

Fig 5. Shows the structure of 45-DEAE-X (4,5-bis-((β -diethylamino)ethoxy)xanthone) and formation of the diprotonated form upon entry of this compound into the parasite digestive vacuole.

DETAILED DESCRIPTION OF THE INVENTION

1. Definitions

The phrase "a compound according to formula X1" and "a xanthone derivative according to formula X1" refer to a compound having the following structure:



wherein:

A is oxygen, substituted antimony (stibium), sulfur or N-R' wherein R' is H, OH, alkyl, haloalkyl, preferably lower alkyl or lower haloalkyl wherein "lower" means 10 or fewer carbon atoms, aryl or haloaryl; and

R_1 - R_8 are independently selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, substituted alkyl (such as alkoxy, alkylamine, alkylthio and haloalkyl), amino, ester and nitro groups and O-linked and C-linked carbohydrates. In alternative
 5 embodiments, an alkyl substituent is a lower alkyl.

References to compounds such as "an X5 compound" refer to the compounds shown in the Summary of the Invention section above. Where a particular substituent
 10 in the formula is intended, it is given parenthetically. For example X5 (A=O) refers to a compound according to formula X5 wherein the A substituent is oxygen, i.e., 2,3,4,5,6-pentahydroxyxanthone.

Compounds of the present invention which may be used to inhibit heme polymerization (and therefore to treat certain parasitic diseases such as malaria) are referred to as "formula XH compounds". A formula XH
 15 compound is a compound broadly defined as:

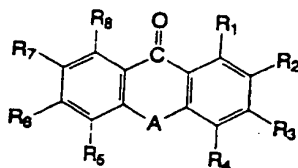


20 wherein X is a group capable of interacting with the iron atom in heme;

Y is a planar aromatic system capable of interacting with the porphyrin ring of heme through overlapping pi-pi orbitals; and

25 Z represents one or more groups capable of interacting with at least one carboxylate side group of heme.

In preferred embodiments, a formula XH compound has the following structure:



30 wherein:

A is oxygen, substituted antimony (stibium), sulfur or N-R' wherein R' is H, OH, alkyl, haloalkyl, preferably lower alkyl or lower haloalkyl wherein "lower" means 10 or fewer carbon atoms, aryl or haloaryl;

R₁-R₃ and R₆-R₈ are independently selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, substituted alkyl (such as alkoxy, alkylamine, alkylthio and haloalkyl), amino, ester and nitro groups and O-linked and C-linked carbohydrates;

at least one member of the R₄ and R₅ substituent pair (and preferably both) is selected from the group consisting of amino, substituted amino, alkylamino, substituted alkyl amino, arylamino, amidinium, alkylamidinium, guanidinium, alkylguanidinium, hydroxy, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy groups, COOH, alkyl-COOH, CONH₂ and alkyl-CONH₂; and

the other member of the R₄ and R₅ substituent pair is selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, substituted alkyl (such as alkoxy, alkylamine, alkylthio and haloalkyl), amino, substituted amino, ester and nitro groups, O-linked and C-linked carbohydrates, alkylamino, substituted alkyl amino, arylamino, amidinium, alkylamidinium, guanidinium, alkylguanidinium, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy groups, COOH, alkyl-COOH, CONH₂ and alkyl-CONH₂.

As used herein, the term "alkyl" encompasses alkanes, alkenes and alkynes, including branched forms, isomers and stereoisomers. In certain embodiments, an alkyl is a lower alkyl, meaning an alkyl having 10 or fewer carbon atoms.

The terms "ester" and "esterification" are used herein as ordinarily understood in the chemical arts,

see, for example, Morrison and Boyd, Organic Chemistry, Allyn & Bacon, Inc., Boston, 1983, herein incorporated by reference. Thus, an ester may be formed by, for example, the combination of an alcohol and an organic acid, with the concurrent elimination of water. The process of forming an ester is termed "esterification." For example, the formula X1 compound 2,3,4,5,6-pentahydroxyxanthone may be esterified by reaction with appropriate acid anhydrides resulting in the net replacement of one or more hydroxyl substituents with ester substituents including, but not limited to: acetoxyl (OCOCH_3); propionyloxy ($\text{OCOCH}_2\text{CH}_3$); and butyryloxy ($\text{OCO}(\text{CH}_2)_2\text{CH}_3$) substituents. Esters produced in this manner may be generally represented by the formula $\text{OCO}(\text{CH}_2)_n\text{CH}_3$, wherein n is zero or a positive integer. In particular embodiments, the term "ester" as used herein refers to an ester wherein n is 1-10.

A "microbial pathogen" is a microorganism capable of causing disease in an animal. The term "microbial pathogen" includes bacterial, mycoplasmal, fungal, helminth and protozoan organisms. "Protozoan parasites" are a subclass of microbial pathogens, being protozoan organisms that are capable of invading, colonizing and, under appropriate conditions, causing disease in an animal. Examples of protozoan parasites include *Leishmania donovani*, *Plasmodium falciparum*, *Giardia lamblia*, *Trypanosoma gambiense* and *Trypanosoma cruzi*. See generally, Robbins et al., Pathologic Basis of Disease (Saunders, 1984) 273-75, 360-83.

A "microbial infection" is a disease caused by a microbial pathogen.

A compound having "anti-microbial activity" is a compound that is capable of inhibiting the growth of a microbial pathogen as determined in *in vivo* or *in vitro* assays of the kind normally employed to determine minimum inhibitory concentrations (MICs) or 50% inhibitory concentrations (IC_{50}) of an antimicrobial agent.

An "oxidant agent" is an agent having the ability to produce or liberate free radical oxygen species or to render parasites or their host cells more susceptible to oxygen radical attack, or having the capacity of oxidizing another compound. Examples of oxidant agents in this general sense include ascorbic acid, hydrogen peroxide, primaquine (or its metabolites) and gamma radiation.

2. Methods

A. Methods for determining biological activity

The anti-parasitic activity of the compounds of the present invention was determined using two different parasites: *Plasmodium falciparum*, a causative agent of malaria; and *Leishmania donovani*, a causative agent of leishmaniasis. The activity of the compounds against yeast was determined using *Candida albicans*.

i. Assay for anti-malarial activity

The D6 strain of *P. falciparum* was cultured in Group A⁺ human erythrocytes and suspended at a 3.3% hematocrit in RPMI-1640 (Gibco, Grand Island, NY) (containing 4g/L glucose, 50mg/L gentamicin and 10% group A⁺ human serum), buffered with 25mM HEPES and 25 mM NaHCO₃ (Trager and Jensen, 1976). Cultures were maintained at 37°C in a gas mixture of 5% oxygen, 5% CO₂, and 90% nitrogen.

The *in vitro* anti-malarial activities of 2,3,4,5,6-pentahydroxyxanthone and other formula X1 compounds were measured by the [³H]-ethanolamine incorporation method as described in Elabbadi et al., 1992, with minor modifications. [³H]-ethanolamine was obtained from American Radiolabeled Chemicals, Inc., St. Louis, MO. Experiments were conducted in 96 well plates in a total volume of 200μl at a final red blood cell concentration of 3.3% (v/v). An initial parasitemia of 0.2 to 0.5% was attained by addition of normal uninfected red cells. Radiolabeled ethanolamine was added after 48 hours of incubation and the experiment

was terminated after 72 hours by collecting the cells onto glass fiber filters with an automated multiwell harvester.

Stock solutions of the various formula X1 compounds were dissolved in DMSO at a concentration of 1 mM and diluted in complete medium (including 10% human serum) to provide 10X stock concentrations in the range of 1 to 10,000 nM. The concentration of the formula X1 compound giving 50% inhibition of label incorporation (IC_{50}) relative to control (i.e., drug-free) conditions was calculated from the dose-response curve.

ii. Assay for anti-leishmania activity

Leishmania donovani was cultivated in Schneider's medium (Gibco, Grand Island, NY) according to the methods described by Grogl et al. (1992). The in vitro susceptibility of *L. donovani* to formula X1 compounds was determined using the radiolabeled thymidine uptake assay essentially as described by Grogl et al. (1992). Briefly, promastigotes were cultivated at 25°C in Schneiders medium supplemented with 20% inactivated fetal calf serum and 100 µg/mL of gentamicin. Cells were maintained in log phase by seeding at 1×10^6 /mL with subculturing when cultured densities approached 4×10^7 /mL before reaching their maximal density. For the assay, early log phase promastigotes were counted on a hemacytometer and resuspended at a concentration of $1-2 \times 10^6$ cell/mL in assay media (Schneiders medium plus 10% fetal bovine serum). Ten-fold serial dilutions of each test compound were prepared as described above and added to 180 µL of the parasite suspension. After incubation for 24 hours at 25°C, methyl-³H-thymidine was added to each sample for a final concentration of 1-2 µCi per well. Each sample was then incubated for an additional 18 hours prior to harvesting. After this incubation time, each sample was aspirated onto a filter mat, washed thoroughly with deionized water, dried and then counted in a scintillation counter with scintillation cocktail.

iii. Assay for anti-Candida activity

The minimum inhibitory concentration (MIC) of formula X1 compounds against a clinical isolate of *Candida albicans* was determined using the following method. As used herein MIC represents the concentration of formula X1 compound that completely inhibits growth of *Candida albicans* over the course of a 15-18 hour incubation period. The determination of this concentration is made by visual inspection; there is no visible growth in a tube containing the MIC of the formula X1 compound whereas visible growth is present in tubes containing sub-MIC concentrations of the compound.

Candida albicans was grown to midlog-phase in Luria-Bertani broth (10 grams Bacto-tryptone, 5 grams Bacto-yeast extract and 10 grams NaCl per liter) and then inoculated into sterile test tubes containing LB broth to an initial density of 10^3 /ml. The formula X1 compound to be tested is dissolved in dimethylsulfoxide (DMSO) at a concentration of 10mM and added to each tube at serial dilutions ($1\mu\text{M}$, $10\mu\text{M}$, $25\mu\text{M}$, $50\mu\text{M}$, $100\mu\text{M}$ and $0\mu\text{M}$). The tubes are incubated at 35°C for 15-18 hours and then visually inspected.

B. Heme Assaysi. In Vitro Heme Polymerization Assay

Heme polymerization was carried out in 0.02 M phosphate buffer, pH 5.2 at 37°C in the absence of proteins. A 10 mM stock solution of hemin chloride in 0.1 M NaOH was prepared freshly and incubated at 37°C for at least 1 hour to effect complete dissolution. Xanthenes were dissolved in dimethylformamide at 10 mM and diluted into 10 ml of pre-warmed phosphate solution to a final concentration of $25\mu\text{M}$. Polymerization was initiated by addition of $25\mu\text{l}$ of the hemin stock solution to the test sample to yield a final concentration of $25\mu\text{M}$ heme. $25\mu\text{l}$ of dimethylformamide was added to the control sample. After 7, 30, 60, 120 and 210 minutes of incubation at 37°C , a 1 ml aliquot was withdrawn, transferred into an Eppendorf tube, and

centrifuged at 14000g for 2 minutes at room temperature to pellet the precipitate. The soluble fraction was then transferred to a semi-microcuvette (polymethylacrylate, VWR), and its absorption was measured at 360 nm against a blank of the test compound in buffer. Control experiments indicated that (i) the pH of the phosphate solution did not change upon addition of the reagents or during the polymerization process, and (ii) the amount of dimethylformamide used in this assay did not significantly affect the rate of polymerization. To estimate the effect of test compounds on heme polymerization at a given time of incubation, the percentage of soluble hemin remaining in the sample was calculated using the following formula:

$$\% \text{ sol. hemin} = [A_{(\text{drug+hemin})t} - A_{(\text{drug})t}] / [A_{(\text{hemin})t=0}] \times 100\%$$

where $A_{(\text{drug+hemin})t}$ is the absorption (360 nm) of the soluble fraction in the drug-hemin sample after various times of incubation; $A_{(\text{drug})t}$ is the absorption of the drug alone; and $A_{(\text{hemin})t=0}$ is the absorption of the hemin control sample (25 μM) measured immediately upon addition of the hemin stock solution.

The dose-dependent inhibition of heme polymerization was evaluated as described above except the concentration of each drug was varied in the range of 0 to 1 mM. The reactions were allowed to proceed for 2 hours in a 37°C waterbath. After incubation, the polymer was pelleted as described above and the absorption (360 nm) of each soluble fraction was measured against a blank containing the drug alone in buffer. The IC_{50} values were determined by nonlinear regression analysis of the dose-response curves of percent inhibition of heme polymerization vs. drug concentration.

3. Production of 2,3,4,5,6-pentahydroxyxanthone
in parasitized erythrocytes treated with
rufigallol and exifone

As disclosed in Winter et al. (1996), rufigallol
5 (1,2,3,5,6,7,-hexahydroxy-9,10-anthraquinone) is a
potent anti-parasitic agent and, when rufigallol is
combined with exifone (2,3,3',4,4',5'-
hexahydroxybenzophenone), a synergistic effect is
observed. The synergy between rufigallol and exifone is
10 noted to produce about a 350-fold increase in potency
against malaria *Plasmodium* parasites.

One aspect of the present invention is the
discovery that rufigallol and exifone interact in the
parasitized erythrocyte to yield 2,3,4,5,6-
15 pentahydroxyxanthone, and that this compound is a potent
anti-malarial agent. Fig. 1 shows a possible mechanism
by which 2,3,4,5,6-pentahydroxyxanthone could be
produced when rufigallol and exifone are present in a
parasitized erythrocyte. Basically, rufigallol is
20 proposed to enter the parasitized erythrocyte, leading
to the formation of hydrogen peroxide in a manner
similar to the well-documented redox cycling behavior of
hydroxynaphthoquinones. In the presence of large
quantities of adventitious iron or iron chelates, such
25 as heme, (liberated as a result of the *Plasmodium*
parasite digesting hemoglobin, Atamna and Ginsburg,
1993), hydrogen peroxide is readily decomposed to
hydroxyl radicals (Goldstein et al., 1993; Aust et al.,
1985). These highly reactive radicals are proposed to
30 attack exifone and transform the diphenyl compound into
2,3,4,5,6-pentahydroxyxanthone.

As reported in U.S. application serial No.
08/520,694, the anti-malarial activity of exifone can be
potentiated by a very wide range of oxidant agents,
35 including ascorbic acid, artemisinin and doxorubicin.
This observation is consistent with the mechanism
proposed above. The production of 2,3,4,5,6-
pentahydroxyxanthone in the proposed reaction scheme was
confirmed by incubating exifone with ascorbic acid in

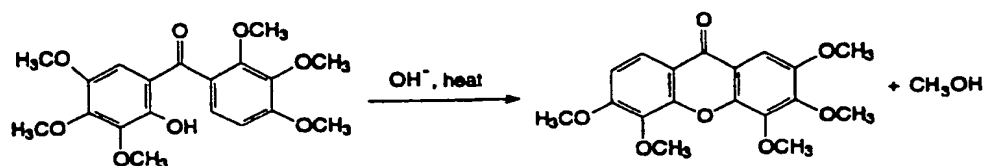
the presence of iron salt and oxygen in a buffered solution at 37-40°C (the "Udenfriend system," Brodie et al., 1954; Maisant et al., 1983; Udenfriend et al., 1954). Samples were removed from the reaction at various time points, lyophilized and extracted with acetone. The solubilized products were then methylated by addition of excess potassium carbonate and dimethylsulfate in acetone and analyzed by gas chromatography-mass spectrometry. A peak corresponding to the methoxy derivative of 2,3,4,5,6-pentahydroxyxanthone was detected.

4. Synthesis and anti-microbial activity of 2,3,4,5,6-pentahydroxyxanthone

The formula X1 compound 2,3,4,5,6-pentahydroxyxanthone is produced using the following method.

A mixture of 1,2,3-Trimethoxybenzene (1.48g) and 2-hydroxy-3,4,5-trimethoxybenzoic acid (2.00g) is stirred in 40ml of ~9% solution of P₂O₅ in methanesulfonic acid at room temperature in a stoppered flask for 4 hours. The 2-hydroxy-3,4,5-trimethoxybenzoic acid was obtained by the method of Mayer and Fikentscher (Mayer and Fikentscher (1956) Chem. Ber. 89:511) from 3,4,5-trimethoxybenzoic acid by bromination and then copper-catalyzed replacement of bromine (by OH) of 2-bromo-3,4,5-trimethoxybenzoic acid. The resultant orange mixture is poured onto crushed ice (500ml) producing an unfilterable gummy precipitate. This crude product is then subjected to base-catalyzed ring closure by heating in a beaker in 100ml of 40% ethanol and 10ml of 10N NaOH just below boiling point. As the mixture reaches 80°C, a white flocculent product appears. The temperature is maintained just below the boiling point and the volume is kept constant by addition of water. After 5 hours, the supernatant is bright yellow and a mass of the precipitate has formed. Heating is continued for 4 more hours. Cooling,

filtering (by suction) and washing with water afforded 1.37g of analytically pure 2,3,4,5,6-pentamethoxyxanthone (yield approximately 45% relative to benzoic acid). This base-catalyzed ring closure is illustrated below:



2,3,4,5,6-pentahydroxyxanthone is then obtained by boron tribromide treatment (200 ml of a 0.8 M solution in CH_2Cl_2) as the pentamethyl ether (0.45 g) is stirred at room temperature for 24 hours. After this period, the solution is poured into 100 ml of water and stirred for approximately 45 minutes before the precipitate is collected by centrifugation. The supernatant is then decanted, the precipitate is shaken with water and centrifuged again. The final product is obtained by freeze-drying of the wet precipitate to produce an orange powder (0.290g, 81%).

The anti-malarial activity of 2,3,4,5,6-pentahydroxyxanthone was determined by the method described above. The IC_{50} was determined to be 0.4-0.5 μM . Chloroquine, a standard anti-malarial agent has an IC_{50} in this assay system of approximately 0.02 μM .

The anti-leishmanial activity of 2,3,4,5,6-pentahydroxyxanthone was determined by the method described above. The IC_{50} was determined to be approximately 5 μM or 0.001 mg/ml. Mangostin, a naturally occurring xanthone, exhibited an IC_{50} of 1 μM (or 0.00041 mg/ml) in this same system. Grögl et al. (1992) report that two commonly used anti-leishmanial drugs, Pentostam and Glucantime, have IC_{50} values in the range of approximately 0.1 - 2 mg/ml.

The MIC of 2,3,4,5,6-pentahydroxyxanthone against *Candida albicans*, determined using the method

described above was found to be approximately 37.5 μ M. This corresponds to an IC_{50} of approximately 10 μ g/ml.

5. Synthesis and anti-microbial activity of
 2,3,4,5,6-pentaacetoxyxanthone

5 Although 2,3,4,5,6-pentahydroxyxanthone was
found to have potent anti-malarial activity, the
inventors postulated that the highly acidic nature of
the 3 and 6 hydroxy groups of this compound (i.e. the R₃
10 and R₆ positions as shown in formula X1) could lead these
groups to be highly ionized at physiological pH values.
Such ionization would likely reduce the rate at which
the compound could cross biological membranes, thereby
lowering the uptake of the compound into parasitized
15 erythrocytes. Accordingly, two derivatives of
2,3,4,5,6-pentahydroxyxanthone were produced which were
expected to be more stable and uncharged above neutral
pH: a pentacetox (i.e. esterified) derivative,
2,3,4,5,6-pentaacetoxyxanthone, as well as a methoxy
20 (i.e. methyl ether) derivative, 2,3,4,5,6-
pentamethoxyxanthone. The activity of these two
derivatives against *P. falciparum* was measured.

As shown in Table 1, the addition of the ether
(methoxy) groups essentially eliminated the anti-
25 malarial activity of the compound, resulting in an IC_{50}
of >100 μ M. This reduction in activity is believed to
be attributable to the extreme stability of the methoxy
groups; the methoxy group is less amenable to enzymatic
cleavage under physiological conditions.

30 The pentaacetox derivative was produced by
heating 2,3,4,5,6,-pentahydroxyxanthone in acetic
anhydride in the presence of a catalytic amount of
sulfuric acid, followed by recrystallization. In
contrast to the methoxy derivative, the esterified
35 2,3,4,5,6,-pentaacetoxyxanthone was several times more
potent than 2,3,4,5,6-pentahydroxyxanthone (exhibiting
an IC_{50} of approximately 0.075 μ M). The enhanced
activity of the esterified compound is postulated to be
due to the ability of the compound to cross membranes

(due to its neutral charge at physiological pH). Esters are also known to be amenable to enzymatic cleavage under physiological conditions. Accordingly, it is expected that the pentaacetoxyxanthone enters the cell
5 where it is enzymatically cleaved to produce pentahydroxanthone.

6. Synthesis and anti-microbial activity of
2,3,4,5,6,7,-hexahydroxanthone

10 The newly discovered anti-malarial activity of 2,3,4,5,6-pentahydroxanthone prompted the investigation of other xanthenes and related compounds. One such related compound was 2,3,4,5,6,7,-hexahydroxanthone which was prepared using the
15 following method:

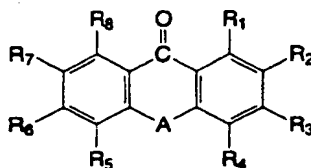
2-hydroxy-3,4,5-trimethoxybenzoic acid (1.14g, 0.005 mol) and 1,2,3,4-tetramethoxybenzene (0.99g, 0.005 mol) and 25 ml of a 9% solution of P_2O_5 in methanesulfonic acid were shaken in a 50ml cylindrical
20 glass tube with a Teflon-lined screw-cap at room temperature for 54 hours. The dark orange mixture was then poured onto crushed ice (150 ml). After melting, the product was extracted with methylene chloride (3 x 40 ml). After removal of the solvent, the residue
25 was chromatographed on silica gel (30 g) with CH_2Cl_2 . Of the three fractions obtained (the eluent was monitored by thin-layer chromatography), the middle one was uniform and left pure 2-hydroxy-,3,4,5,2',3',4',5'-heptamethoxybenzophenone (0.51 g, 25%) as a yellow oil
30 upon evaporation of the solvent. This was dissolved in 100 ml 75% alcohol whereafter 5 ml of 10N NaOH were added and the mixture was heated to boiling in a beaker for three hours; the volume was kept at 100 ml by occasional addition of water. The mixture was then
35 transferred to a 250 ml round bottom flask and refluxed for another 17 hours. After cooling, suction filtration yielded 0.36 g of 2,3,4,5,6,7-hexamethoxyxanthone as a white product (small needles, matted, 77%). In a

deprotection procedure, similar to the one described above for pentamethoxyxanthone, 0.42 g of the hexamethoxyxanthone produced 0.296 g of hexahydroxyxanthone (91%) as a pale yellow powder. It was found advantageous to circumvent the need for centrifugation by stirring the methylene chloride-water mixture (from the quenching of the BBr_3 -solution) in a wide-mouthed container for several hours, leading to the evaporation of the methylene chloride; the mixture is then easily filterable.

The antimalarial activity of 2,3,4,5,6,7-hexahydroxyxanthone was determined by the method described above. The IC_{50} was determined to be $0.075 \mu\text{M}$. The IC_{50} of this compound against *Leishmania* was determined to be approximately $5 \mu\text{M}$. The MIC of the compound against *Candida albicans* was determined to be approximately $37.5 \mu\text{M}$, corresponding to an IC_{50} of approximately $10 \mu\text{g/ml}$.

7. Scope of formula X1 compounds

The inventors have discovered that a wide range of compounds related to 2,3,4,5,6-pentahydroxyxanthone have anti-microbial activity. These compounds can be represented by the formula X1 shown below.



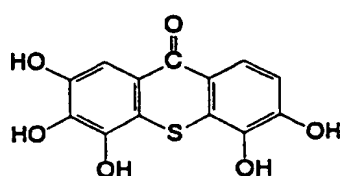
wherein:

A is oxygen, substituted antimony (stibium), sulfur or N-R' wherein R' is H, OH, alkyl, haloalkyl, aryl or haloaryl; and

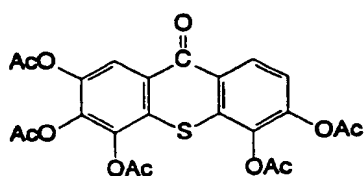
R_1 - R_8 are independently selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, substituted alkyl (such as alkylamine, alkylthio and

haloalkyl), amino, ester and nitro groups and O-linked and C-linked carbohydrates.

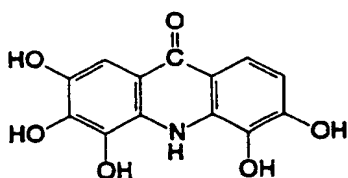
The activities of various formula X1 compounds against the *Plasmodium falciparum* parasite are shown in Table 1. The activities of various formula X1 compounds against *Leishmania donovani* are shown in Table 2. For comparison, Table 2 also shows the activity of stibogluconate, a standard anti-leishmanial. Other examples of specific formula X1 compounds are illustrated below:



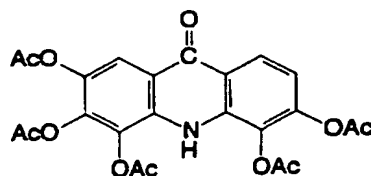
2,3,4,5,6-pentahydroxythioxanthone



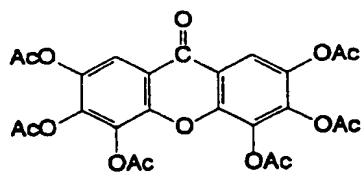
2,3,4,5,6-pentaacetoxythioxanthone



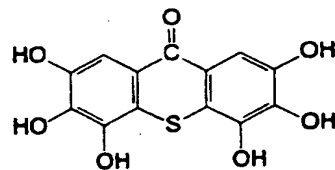
2,3,4,5,6-pentahydroxyacridone



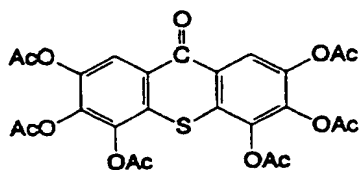
2,3,4,5,6-pentaacetoxyacridone



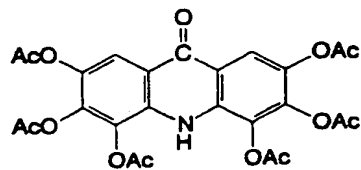
2,3,4,5,6,7-hexaacetoxyxanthone



2,3,4,5,6,7-hexahydroxythioxanthone

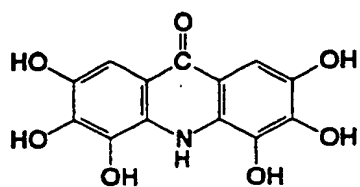


2,3,4,5,6,7-hexaacetoxythioxanthone

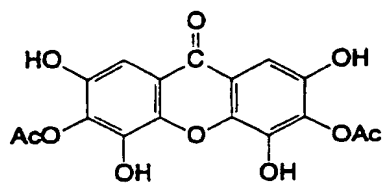


2,3,4,5,6,7-hexaacetoxyacridone

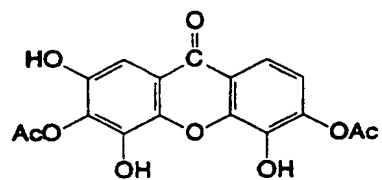
5

**2,3,4,5,6,7-hexahydroxyacridone**

10

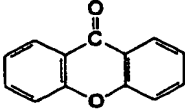
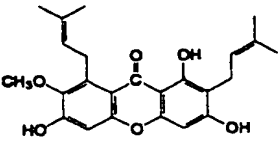
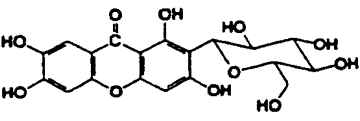
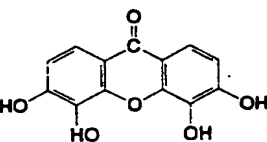
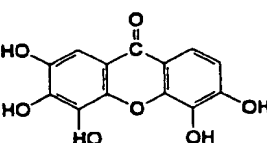
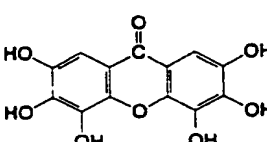
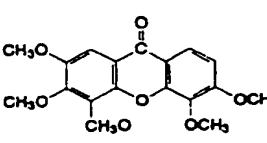
**2,4,6,7-tetrahydroxy-3,6-acetoxyanthrone**

20

**2,4,5-trihydroxy-3,6-acetoxyanthrone**

25

Table 1

Compound Name	Xanthone Structure	IC ₅₀ , μ M vs. <i>Plasmodium</i>
Xanthone		> 10
Mangostin		5
Mangiferin		50
3,4,5,6,- Tetrahydroxy- xanthone		10
2,3,4,5,6- Pentahydroxy- xanthone		0.4 to 0.5
2,3,4,5,6,7- hexahydroxy- xanthone		0.075
2,3,4,5,6- Pentamethoxy- xanthone		> 100

5

10

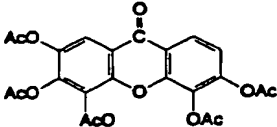
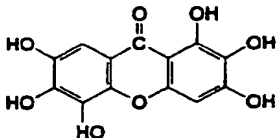
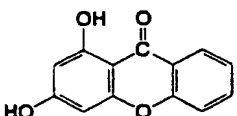
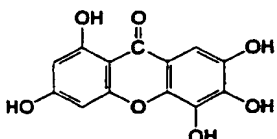
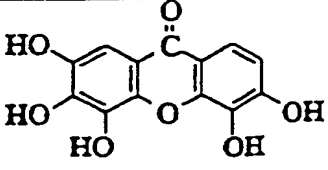
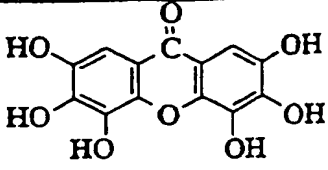
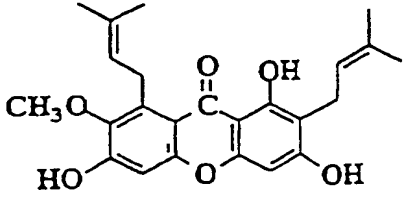
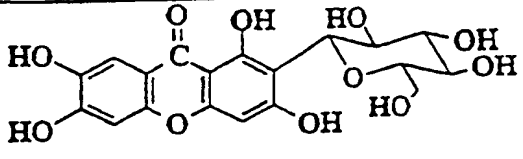
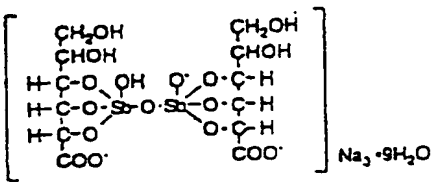
Compound Name	Xanthone Structure	IC ₅₀ , μ M vs. <i>Plasmodium</i>
2,3,4,5,6-Penta-acetoxyxanthone		0.075
1,2,3,5,6,7-Hexahydroxyxanthone		25-50
1,3-dihydroxyxanthone		> 100 μ M
1,3,5,6,7-pentahydroxyxanthone		1 μ M

Table 2

Compound	IC ₅₀ , mg/ml vs. Leishmania
 2,3,4,5,6-Pentahydroxyxanthone "X5"	0.0015
 2,3,4,5,6,7-hexahydroxyxanthone "X6"	0.0015
 Mangosin	0.00041
 Mangiferin	>0.05
 Sodium Antimonyl (V) Gluconate "Stibogluconate" Stibogluconate	0.1 to 1.0*

*taken from literature values

8. Sources of formula XI compounds and preferred method of synthesis

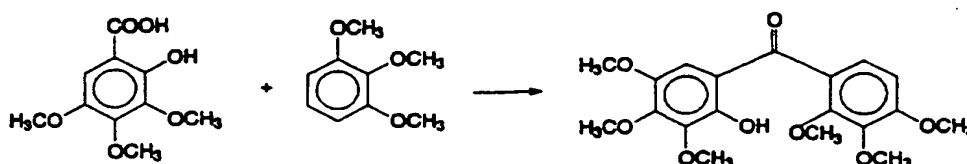
Many xanthenes and xanthone derivatives can be purchased commercially from sources including: ICN Biomedicals, Irvine, California, U.S.A.; Sigma Chemical Company, St. Louis, Missouri, U.S.A.; Aldrich Chemical Company, Milwaukee, Wisconsin, U.S.A.; and Janssen Chimica (Belgium). In addition, many xanthenes are naturally occurring compounds which can be purified by methods such as those described in Hostettmann et al. (1995).

A. General method of xanthone synthesis

Xanthenes according to the present invention may be synthesized by the general method described above, for the synthesis of 2,3,4,5,6,7-hexahydroxyxanthone and 2,3,4,5,6-pentahydroxyxanthone. Essentially, this method comprises subjecting an o-hydroxy-o'-methoxy-benzophenone to base treatment (e.g., aqueous sodium hydroxide), which leads to the formation of the central oxygen-bridged ring; the o-phenoxide (from the o-hydroxyl in basic medium) then replaces the methoxide on the other ring by nucleophilic substitution. The net effect is expulsion of CH_3O^- , and the formation of a diphenyl ether. Since the two phenyl rings are already linked by a carbonyl group, a xanthone is obtained. The o-OH, o'-OCH₃ groupings are required for this reaction; although the methyl could be replaced with other groups, this is not likely to be of any advantage since methyl ethers are readily available. However, other substituents can be present in the two aromatic rings of the benzophenones. For example, for the synthesis of the penta- and hexa-hydroxyxanthenes described above, these other substituents were methoxy groups.

The benzophenones used in synthesizing the xanthenes as described above may be obtained by combining substituted benzoic acids and methoxybenzenes by a condensation or other coupling procedure. In an

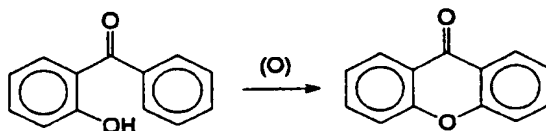
exemplary condensation procedure, the benzoic acid carries an o-hydroxy group:



This coupling can be achieved by condensation in polyphosphoric acid or a mixture of phosphorus pentoxide and methanesulfonic acid. Alternatively, benzophenones may be synthesized by Friedel-Crafts acylation (of a benzoyl chloride and a polymethoxybenzene), or by the Hoesch synthesis, or by reaction of a benzoyl chloride with an appropriately metalated (e.g., lithiated) aromatic.

In particular cases, additional substituents may be introduced into the benzophenone after the benzophenone has been synthesized.

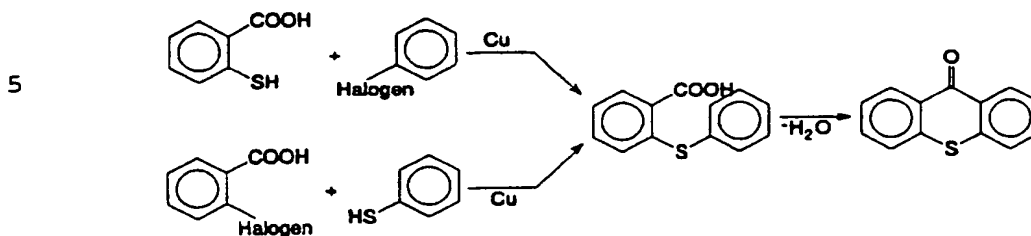
Alternatively, xanthenes may be derived from benzophenones by oxidative cyclization. This method essentially requires an o-hydroxy group on one ring and a free position (occupied by H) on the other ring. Oxidation (e.g., with $K_3[Fe(CN)_6]$, or $KMnO_4$) produces an oxygen bridge with the elimination of $2H$.



B. Synthesis of Thioxanthenes

Thioxanthenes may be obtained by a number of methods. Exemplary syntheses include: (1) combining an o-mercaptobenzoic acid with a halobenzene (preferably iodo or bromo); and (2) combining an o-halobenzoic acid (preferably either bromo or iodo) with a mercaptobenzene. The intermediate diphenylsulfide produced in each case is then condensed to yield the

required thioxanthone as illustrated in the following schematic:



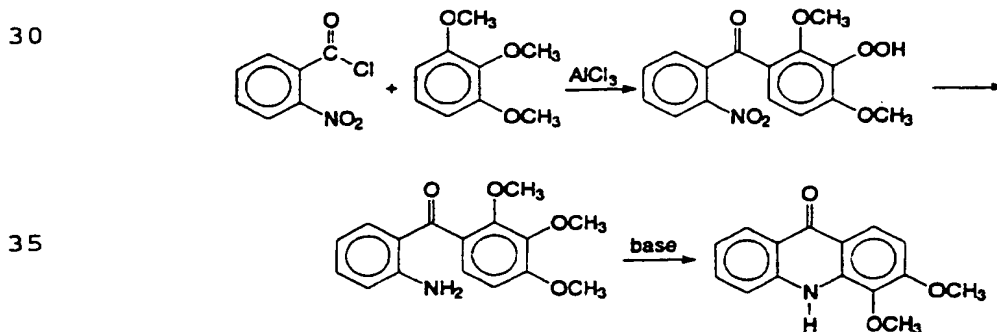
10 Methods of synthesizing thioxanthenes using this general reaction scheme are described in Hollis-Showalter et al., J. Med. Chem. 31, 1527 (1988).

C. Synthesis of Acridones

15 Acridones may be synthesized by a number of different methods. The following methods are exemplary and are well known in the art.

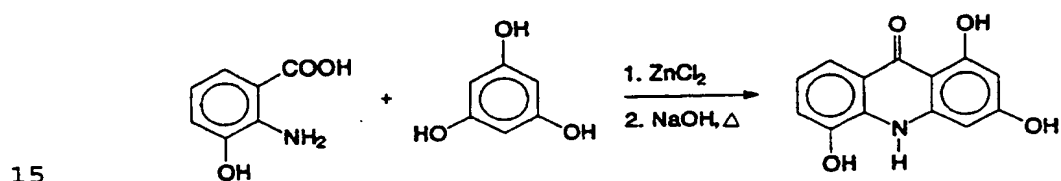
Acridones may be formed from o-nitrobenzophenones, which are reduced to obtain o-aminobenzophenones which are in turn cyclized with either o'-methoxy or o'-hydroxy groups to produce the acridones. The o-nitrobenzophenones which are used as starting materials may be obtained either by Friedel-Crafts acylation of phenols or methoxybenzenes using o-nitrobenzoylchlorides, or by direct nitration of benzophenones, or by coupling of lithiated arenes with o-nitrobenzoyl chlorides (e.g., as described by Parkham et al., Journal of Organic Chemistry 46, 1057 (1981)).

25 An exemplary synthesis is illustrated below:

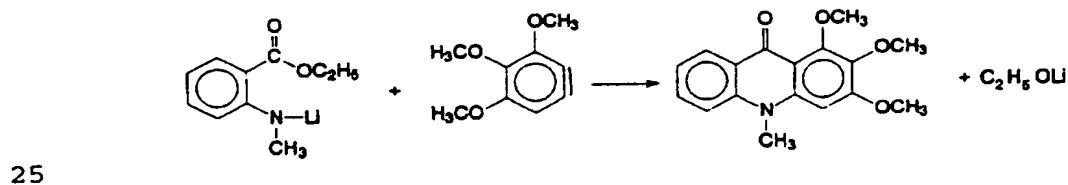


Alternatively, o-nitrobenzophenones may be formed by coupling of 2-methyl-3,1-benzoxazin-4-ones (from o-aminobenzoic acid by heating with acetic anhydride) with aromatic Grignard reagents (e.g., Adams et al. J.C.S. Perkin Trans I 2089 (1976)).

Alternatively, acridones may be produced by zinc chloride catalyzed condensation of hydroxyanthranilic acids and polyhydroxybenzenes (such as described by Bahar et al., Phytochemistry 21, 2729 (1982)) and illustrated in the following scheme:



Acridones may also be formed by cycloaddition of derivatives of anthranilic acids with dehydrobenzenes such as described by Khanapure et al., Tetrahedron Letters 31:2869 (1990).



D. Deprotection

Deprotection of polymethoxyxanthenes, polymethoxythioxanthenes or polymethoxyacridones may be achieved in a number of ways, including treating with either hydriodic acid or with a methylene chloride solution of boron tribromide, and hydrolysis of the intermediate boron-phenoxy compound.

35

9. Activity of formula X1 compounds

The formula X1 compounds according to the present invention are useful in inhibiting the growth of microbial pathogens, including protozoan parasites (for example, *Plasmodium* sp. and *Leishmania* sp.) and yeast (for example, *Candida albicans*). Thus, one aspect of the present invention is a method of inhibiting the growth of a microbial pathogen by contacting the microbial pathogen with a formula X1 compound. In this context, it is, of course, necessary to contact the microbial pathogen with a sufficient amount of the formula X1 compound to inhibit growth of the pathogen. One skilled in the art will readily appreciate that the amount of compound sufficient to inhibit the growth of the microbial pathogen will vary according to the formula X1 compounds selected, the target microbial pathogen and the environment in which the microbial pathogen is growing. Standard methods are available for determining the IC_{50} concentration of formula X1 compounds for microbial pathogens *in vitro*. Alternatively, ED_{50} values may be determined in an animal. See Munson, Principles of Pharmacology (Chapman and Hall, 1995) Chapter 1. Exemplary IC_{50} values (showing activities against *Plasmodium* and *Leishmania*, respectively) are presented in Tables 1 and 2. These values relate to the inhibition of a microbial pathogen grown *in vitro*. Contacting the microbial pathogen with a compound according to formula X1 may also be performed *in vivo* where necessary to inhibit the growth of microbial pathogens under physiological conditions. Section 11 below ("Pharmaceutical Compositions") addresses compositions and dosages appropriate for inhibiting the growth of microbial pathogens in such circumstances.

35 10. Heme Polymerization and Formula XH Compounds

Certain parasites, including *Plasmodium* spp. and *Schistosoma* spp., obtain amino acids for growth by degrading hemoglobin from the red blood cells of the

infected host. In the case of the malarial parasite, degradation of hemoglobin takes place in the parasite's digestive vacuole, which is an acidic proteolytic compartment essential to the metabolism of the parasite (see Fig. 2). As the hemoglobin is broken down, free toxic heme is released. To prevent the build-up of toxic heme, the parasites polymerize the heme for storage in a non-toxic form called hemozoin.

While the formula X1 compounds of the present invention exhibit anti-microbial activity against a range of pathogens, it has now been discovered that a certain sub-group of these compounds form complexes with free heme, which results in the inhibition of heme polymerization. In turn, this leads to the accumulation of toxic heme in the parasite's digestive vacuole and ultimately to the death of the parasite. These compounds, include X5 (A=O) and X6 (A=O) and belong to a group related to formula X1 compounds which are referred to herein as "formula XH compounds". Formula HX compounds may be particularly effective against those parasites, including *Plasmodium* and *Schistosoma*, which rely on hemoglobin catabolism to survive in the infected host or must rely on the host's heme iron reserves for synthesis of critical ferroproteins.

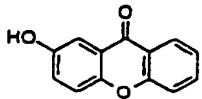
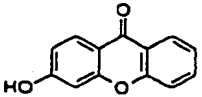
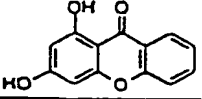
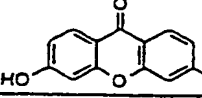
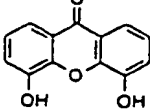
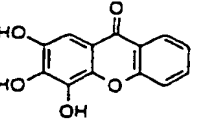
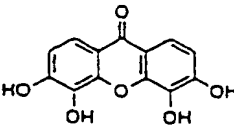
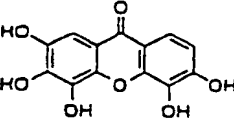
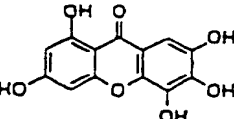
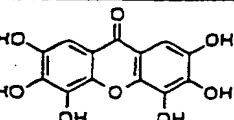
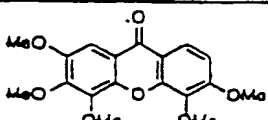
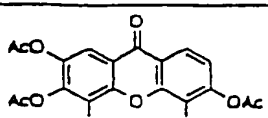
Table 3 below shows the ability of a range of formula X1 compounds to inhibit heme polymerization, as determined using the simple in vitro heme polymerization assay described above. Under the conditions of this assay, heme polymerization was found to be pH dependent (polymerization required a pH of between 4.5 and 5.5). Polymerization occurred spontaneously and was more than 95% complete within 2 hours of commencement of incubation with the compound X5 (A=O) (see Fig. 3).

The IC_{50} values shown in Table 3 are the average of at least two independent determinations of full dose-response curves. Xanthone and the tested monohydroxyxanthenes did not exhibit any inhibitory activity in this assay. Moderate inhibitory activity

(i.e., IC_{50} 8-20 μM) was observed for the compounds bearing a single hydroxy group at either 4- or 5-position, whereas the greatest activity was observed for xanthenes containing hydroxy groups at both positions.

- 5 For example, 2,3,4-trihydroxyxanthone exhibited an IC_{50} of 16.5 μM , while 2,3,4,5,6-pentahydroxyxanthone (X5, A=O) yielded a value of 1.2 μM . Consistent with this structure-activity profile, the 4,5-hydroxylated
- 10 xanthenes also exhibited the most pronounced in vitro antimalarial activity. Furthermore, pentamethoxy-X5 and pentaacetyl-X5 were inactive in this assay, though the latter was shown to be a potent antimalarial agent. Presumably, pentaacetyl-X5 is hydrolysable in infected
- 15 red blood cells by a non-specific esterase, whereas pentamethoxy-X5 is not.

Table 3 - 36 -

Compound name	Compound structure	IC ₅₀ μ M, <i>P. falciparum</i> clone D6	IC ₅₀ μ M in vitro heme polymerization
2-hydroxyxanthone		50	>1000
3-hydroxyxanthone		>100	>1000
1,3-dihydroxyxanthone		75	>1000
3,6-dihydroxyxanthone		>100	>500
4,5-dihydroxyxanthone		16	14
2,3,4-trihydroxyxanthone		40	17
3,4,5,6-tetrahydroxyxanthone		5	2.5
2,3,4,5,6-pentahydroxyxanthone (X5)		0.4	1.2
1,3,5,6,7-pentahydroxyxanthone		1	9
2,3,4,5,6,7-hexahydroxyxanthone (X6)		0.1	1.4
2,3,4,5,6-pentamethoxyxanthone		>100	>1000
2,3,4,5,6-pentacetylxanthone		0.075	>1000

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These findings suggest that X5 (A=O) and other compounds shown in Table 3 form soluble complexes with heme monomers or oligomers and interfere with hemozoin formation. Such action may result in the death of the parasite by one of several mechanisms, including preventing detoxification of free heme, starving the parasite for iron, or increasing the osmotic pressure within the parasite digestive vacuole. The relative abilities of these compounds to inhibit in vitro heme polymerization are in good correlation with their in vitro antimalarial activities, and are indicative of the following structure-activity relationships: (i) in general, a higher degree of hydroxylation is favored for the inhibitory activity; and (ii) hydroxylation at 4- and 5-positions appears to be central to full activity. Based on these observations, a model for the interaction of these compounds is presented in Fig. 4. This model shows the interaction of X6 (A=O) with heme and serves to illustrate the following interactions: (1) between the heme iron and the carbonyl oxygen; (2) between the two planar aromatic systems; and (3) between the carboxylate side groups of the heme and the 4- and 5-position hydroxyls of the xanthone. These interactions may take any form of known chemical interaction, including covalent bonding, hydrogen bonding, ionic bonding, and polar and nonpolar bonding. Moreover, this model predicts that chemical modifications at the 4- and/or 5-positions which improve association with the heme carboxylate groups will result in even greater antimalarial activity.

Accordingly, Formula XH compounds can be defined as compounds which inhibit heme polymerization and which have the following structure:

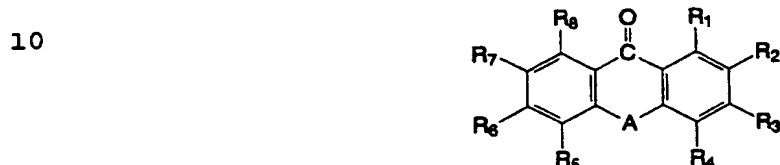


wherein X is a group capable of interacting with the iron atom in heme;

Y is a planar aromatic system capable of interacting with the porphyrin ring of heme through overlapping pi-pi orbitals; and

Z represents one or more groups capable of interacting with at least one carboxylate side group of heme.

In preferred embodiments, a formula XH compound has the following structure:



15 wherein:

A is oxygen, substituted antimony (stibium), sulfur or N-R' wherein R' is H, OH, alkyl, haloalkyl, preferably lower alkyl or lower haloalkyl wherein "lower" means 10 or fewer carbon atoms, aryl or

20 haloaryl;

R₁-R₃ and R₆-R₈ are independently selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, substituted alkyl (such as alkoxy, alkylamine, alkylthio and haloalkyl), amino, ester and nitro groups and O-linked and C-linked carbohydrates;

25 at least one of the R₄ and R₅ substituent pair is selected from the group consisting of amino, substituted amino, alkylamino, substituted alkyl amino, arylamino, amidinium, alkylamidinium, guanidinium, alkylguanidinium, hydroxy, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy groups, COOH, alkyl-COOH, CONH₂ and alkyl-CONH₂; and

30 the other member of the R₄ and R₅ substituent pair is selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, substituted alkyl (such as alkoxy, alkylamine, alkylthio and haloalkyl), amino, substituted amino, ester and nitro groups, O-linked and

C-linked carbohydrates, alkylamino, substituted alkyl amino, arylamino, amidinium, alkylamidinium, guanidinium, alkylguanidinium, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy groups, COOH, alkyl-COOH, CONH₂, and alkyl-CONH₂.

Preferably, both R₁ and R₂ are selected from the group consisting of amino, substituted amino, alkylamino, substituted alkyl amino, arylamino, amidinium, alkylamidinium, guanidinium, alkylguanidinium, hydroxy, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy groups, COOH, alkyl-COOH, CONH₂, and alkyl-CONH₂.

Examples of formula XH compounds include: 4,5-dihydroxyxanthone, , 2,3,4-trihydroxyxanthone, 3,4,5,6-tetrahydroxyxanthone, 2,3,4,5,6-pentahydroxyxanthone, 1,3,5,6,7-pentahydroxyxanthone, 2,3,4,5,6,7-hexahydroxyxanthone. Methods of synthesizing these compounds are known in the art and are described in a representative manner above in relation to formula X1 compounds (it will be appreciated that there is significant overlap between formula X1 and formula XH compounds).

Based on the mechanism proposed for the interaction of formula XH compounds with heme, the formula XH compound 4,5-bis-((β -diethylamino)ethoxy)xanthone (45-DEA-X) is anticipated to be particularly effective in complexing with heme. 4,5-DEAE-X, illustrated in Fig. 5, is a diprotic base which upon entry into the acidic vacuole becomes positively charged, effectively "trapping" the drug within this compartment where it will complex with heme. The positively charged residues are arranged to be in opposition to the heme carboxylate side chains so as to facilitate formation of a soluble heme:xanthone complex (the ionic nature of the trapped xanthone will also

maintain the drug:heme complex in solution). 4,5-DEAE-X may be readily prepared from 4,5-dihydroxyxanthone (which as described above, is synthesized by base-catalyzed cyclization of the appropriate ortho-hydroxy-methoxylated-benzophenone). To produce 4,5-DEAE-X, 5 4,5-Di-hydroxyxanthone is then reacted under basic conditions with ethylene dibromide to yield 4,5-bis-(β -bromoethoxy)-xanthone. The latter is then reacted with diethylamine to yield the desired product. The shorter 10 dimethylaminomethoxy derivative is also easily available from 4,5-dihydroxyxanthone through reaction with Eschenmoser's salt.

The present invention thus encompasses the use of formula XH compounds to inhibit heme polymerization and to inhibit the growth of those pathogens which 15 polymerize heme, such as *Plasmodium*. It is also apparent that the formula XH heme-complexing compounds may be used to treat infections caused by pathogens which require access to the host heme iron reserves for 20 survival.

11. Pharmaceutical compositions

Formula X1 and XH compounds having anti-microbial activity are administered to patients in 25 conventional dosage forms prepared by combining an appropriate dose of the compound with standard pharmaceutical carriers. Suitable pharmaceutical carriers may be, for example, solids or liquids. Suitable solid carriers include lactose, magnesium 30 stearate, terra alba, sucrose, talc, stearic acid, gelatin, agar, pectin, acacia and cocoa butter. The amount of solid carrier will vary widely depending on which carrier is selected, but preferably will be from about 25mg to about 1g. Suitable liquid carriers 35 include syrup, peanut oil, olive oil, sesame oil, propylene glycol, polyethylene glycol and water. The carrier or diluent may also include time delay material well known to the art such as, for example, glyceryl,

monostearate or glycerol distearate, either alone or with a wax. The foregoing examples of suitable pharmaceutical carriers are only exemplary and one of skill in the art will recognize that a very wide range of such carriers may be employed.

The formulation of the formula X1 and XH compounds with a pharmaceutical carrier can take many forms. For example, the formulation may be a tablet, capsule, powder, suppository, lozenge, syrup, emulsion, liquid suspension or solution, or sterile injectable liquid. The pharmaceutical compositions are prepared by conventional techniques involving procedures such as mixing, granulating and compressing, and dissolving the ingredients. As will be appreciated from the foregoing exemplary formulations, administration of the compounds can be by any known route, including oral administration, intramuscular and intravascular injection.

The methods of treating a patient suffering from a microbial disease, such as malaria, in accordance with this invention comprise administering to the patient a therapeutically effective amount of a compound according to formula X1 or formula XH. Preferably, the patient will be administered the compound in a formulation as described above (i.e. in combination with a pharmaceutical carrier), the formulation having a therapeutically effective amount of the compound.

As used herein, "a therapeutically effective amount" is preferably an amount that results in complete remission of the disease. However, it will be recognized that any improvement in the patient's condition is clinically advantageous. Hence, "a therapeutically effective amount" also encompasses amounts of the administered compound that result in partial remission of the disease or which slow or limit the further progression of the disease, or which inhibit the growth of the infectious agent or which reduce the clinical signs and symptoms of the disease (for example,

fever and chills in a malaria infection). It is anticipated that therapeutically effective dosages which slow or limit the spread of the disease, or which inhibit the growth of the parasite will be particularly suitable for combination with other anti-microbial drugs.

The compounds of the invention can be administered in a daily dosage schedule of from about 10 mg to about 10 g. One skilled in the art will recognize that in determining the active amount of the anti-microbial compound to be administered, the activity of the specific compound selection, the age, weight and condition of the patient and the administration of other drugs to the patient must be considered.

The formula X1 and XH compounds may also be indirectly provided to patients in pro-drug formulations. For example, formula X1 and XH xanthenes may be produced by co-administration of an oxidant agent with a corresponding substituted benzophenone under physiological conditions. A pro-drug is thus defined herein as a compound which reacts under physiological conditions to produce a formula X1 or XH compound. Thus, the pro-drug for 4,5-DEAE-X would be 3,3'-bis-(β -diethylamino)ethoxy-2-hydroxy-benzophenone, and the pro-drug for 2,3,4,5,6-pentahydroxyxanthone would be 2,3,3',4,4',5'-hexahydroxybenzophenone (exifone). The provision of the X1 and XH compounds in pro-drug form (i.e. the corresponding benzophenones) may be particularly useful where the oxidant agent which is administered with the pro-drug is another anti-microbial agent. For example, the widely used anti-malarial agent primaquine is such an oxidant agent, and the combination of an XH pro-drug with primaquine is expected to be a particularly efficacious treatment for malaria.

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WHAT IS CLAIMED IS:

1. A compound according to formula X1 or formula XH.

5

2. A composition comprising a compound according to formula 1 in combination with a suitable pharmaceutical carrier.

10

3. A method of inhibiting the growth of a microbial pathogen comprising contacting the pathogen with a sufficient amount of a compound according to claim 1.

15

4. A compound of structure:

X - Y - Z

wherein X is a group capable of interacting with the iron atom in heme;

20 Y is a planar aromatic system capable of interacting with the porphyrin ring of heme through overlapping pi-pi orbitals; and

Z represents one or more groups capable of interacting with at least one carboxylate side group of heme.

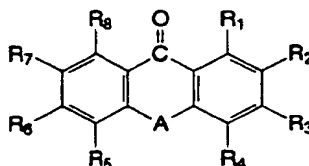
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5. A method of inhibiting the polymerization of heme, comprising contacting heme with a compound according to claim 4.

30

6. A compound of claim 4 wherein the compound is of formula:

35



wherein:

A is oxygen, substituted antimony (stibium), sulfur or N-R' wherein R' is H, OH, alkyl, haloalkyl, preferably lower alkyl or lower haloalkyl wherein "lower" means 10 or fewer carbon atoms, aryl or

5 haloaryl;

R₁-R₃ and R₆-R₈ are independently selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, substituted alkyl (such as alkoxy, alkylamine, alkylthio and haloalkyl), amino, ester and nitro groups
10 and O-linked and C-linked carbohydrates;

at least one of the R₄ and R₅ substituent pair is selected from the group consisting of amino, substituted amino, alkylamino, substituted alkyl amino, arylamino, amidinium, alkylamidinium, guanidinium,
15 alkylguanidinium, hydroxy, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy groups, COOH, alkyl-COOH, CONH₂ and alkyl-CONH₂; and

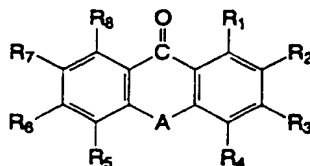
the other member of the R₄ and R₅ substituent pair is selected from the group consisting of H, OH, halogen, aryl, arylamine, alkyl, substituted alkyl (such as alkoxy, alkylamine, alkylthio and haloalkyl), amino, substituted amino, ester and nitro groups, O-linked and C-linked carbohydrates, alkylamino, substituted alkyl
20 amino, arylamino, amidinium, alkylamidinium, guanidinium, alkylguanidinium, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy groups, COOH, alkyl-COOH, CONH₂ and alkyl-
25 CONH₂.
30

7. A compound according to claim 6 wherein the compound is selected from the group consisting of: 4,5-dihydroxyxanthone, 2,3,4-trihydroxyxanthone, 3,4,5,6-tetrahydroxyxanthone, 2,3,4,5,6-pentahydroxyxanthone,
35 1,3,5,6,7-pentahydroxyxanthone, 2,3,4,5,6,7-hexahydroxyxanthone; and 4,5-bis-((β-diethylamino)ethoxy)xanthone.

8. A compound according to claim 6 wherein both R_4 and R_5 are selected from the group consisting of amino, substituted amino, alkylamino, substituted alkyl amino, arylamino, amidinium, alkylamidinium, guanidinium, alkylguanidinium, hydroxy, alkylhydroxy, alkoxyhydroxy, alkoxyamine, alkoxy-substituted amine, azido, carboxylic esters of hydroxy, alkylhydroxy and alkoxyhydroxy groups, COOH, alkyl-COOH, CONH₂, and alkyl-CONH₂.

9. A composition for the treatment of a microbial disease consisting essentially of:

a therapeutically effective amount of a compound having a formula X1:



wherein A is oxygen, substituted antimony (stibium), sulfur or N-R' wherein R' is H, hydroxy, alkyl, haloalkyl, aryl or haloaryl, and R₁-R₈ are independently selected from the group consisting of H, OH, aryl, arylamine, alkyl, substituted alkyl, halogen, amino, ester and nitro groups and O-linked and C-linked carbohydrates; and

a suitable pharmaceutical carrier.

10. A method of inhibiting the growth of a microbial pathogen, the method comprising the steps of: providing a composition according to claim 9; and

contacting the microbial pathogen with the compound.

11. The method of claim 10 wherein the compound is selected from the group consisting of 2,3,4,5,6-

pentahydroxyxanthone; 2,3,4,5,6-pentaacetoxylxanthone;
 2,3,4,5,6,7-hexahydroxyxanthone; 2,3,4,5,6,7-
 hexaacetoxylxanthone; 2,3,4,5,6-pentahydroxyacridone;
 2,3,4,5,6-pentaacetoxylacridone; 2,3,4,5,6,7-
 5 hexahydroxyacridone; 2,3,4,5,6,7-hexaacetoxylacridone;
 2,3,4,5,6-pentahydroxythioxanthone; 2,3,4,5,6-
 pentaacetoxylthioxanthone; 2,3,4,5,6,7-
 hexahydroxythioxanthone; and 2,3,4,5,6,7-
 hexaacetoxylthioxanthone.

10

12. The method of claim 10 wherein the
 microbial pathogen is a protozoan parasite.

13. The method of claim 12 wherein the
 15 protozoan parasite is a *Plasmodium* species or a
Leishmania species.

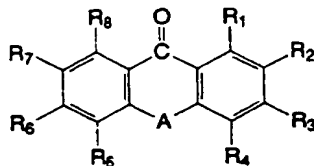
14. The method of claim 10 wherein the
 microbial pathogen is a bacterial pathogen.

20

15. The method of claim 10 wherein the
 microbial pathogen is a fungal species.

16. The method of claim 10 wherein the pathogen
 25 is a Helminth, such as a *Schistosoma* spp.

17. A method of inhibiting the growth of a
 protozoan parasite, the method comprising the steps of:
 providing a therapeutically effective amount of
 30 a composition comprising a compound having a formula



35

wherein A is oxygen, substituted antimony (stibium),
 sulfur, or N-R' wherein R' is H, hydroxy, alkyl,

haloalkyl, aryl or haloaryl, and R_1 - R_8 are independently selected from the group consisting of H, OH, aryl, arylamine, alkyl, substituted alkyl, halogen, amino, ester and nitro groups, and O-linked and C-linked
5 carbohydrates; and

contacting the protozoan parasite with the compound.

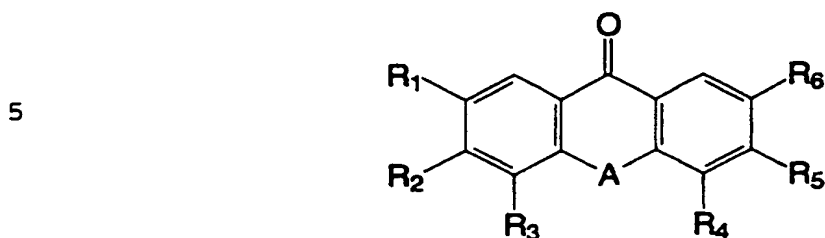
18. The method of claim 17 wherein in the
10 formula R_1 and R_8 are H.

19. The method of claim 17 wherein the step of contacting the parasite with the compound comprises administering a therapeutically effective dosage of the
15 compound to a patient infected with the parasite.

20. The method of claim 17 wherein the protozoan parasite is a *Plasmodium* sp. or a *Leishmania* sp.
20

21. A method of inhibiting the growth of a protozoan parasite, the method comprising contacting the parasite with an amount of a compound selected from the following group, the amount sufficient to inhibit the
25 growth of the parasite: 2,3,4,5,6-pentahydroxyxanthone; 2,3,4,5,6-pentaacetoxyxanthone; 2,3,4,5,6,7-hexahydroxyxanthone; 2,3,4,5,6,7-hexaacetoxyxanthone; 2,3,4,5,6-pentahydroxyacridone; 2,3,4,5,6-pentaacetoxycridone; 2,3,4,5,6,7-hexahydroxyacridone;
30 2,3,4,5,6,7-hexaacetoxycridone; 2,3,4,5,6-pentahydroxythioxanthone; 2,3,4,5,6-pentaacetoxythioxanthone; 2,3,4,5,6,7-hexahydroxythioxanthone; 2,3,4,5,6,7-hexaacetoxythioxanthone; and 4,5-bis-(diethylamino-ethoxy)-xanthone.
35

22. A compound of formula:



wherein A is

10 O, S, substituted Sb or N-R' wherein R' is H, OH, alkyl, haloalkyl, aryl or haloaryl; and

R₁-R₆ are selected from the following options:

(1) R₆ is H and R₂ - R₅ are independently selected from the group consisting of OH, halogen, OCOCH₃, and OCO(CH₂)_nCH₃, wherein n is 1-10; and

15

(2) R₁ - R₆ are independently selected from the group consisting of OH, halogen, OCOCH₃, and OCO(CH₂)_nCH₃, wherein n is 1-5.

20 23. The compound of claim 22 wherein the compound is selected from the group consisting of:
 2,3,4,5,6-pentahydroxyxanthone; 2,3,4,5,6-pentaacetoxyxanthone; 2,3,4,5,6,7-hexahydroxyxanthone;
 2,3,4,5,6,7-hexaacetoxyxanthone; 2,3,4,5,6-pentahydroxyacridone; 2,3,4,5,6-pentaacetoxyacridone;
 2,3,4,5,6,7-hexahydroxyacridone; 2,3,4,5,6,7-hexaacetoxyacridone; 2,3,4,5,6-pentahydroxythioxanthone;
 2,3,4,5,6-pentaacetoxythioxanthone; 2,3,4,5,6,7-hexahydroxythioxanthone; and 2,3,4,5,6,7-hexaacetoxythioxanthone.

25

30

24. A therapeutic composition for inhibiting the growth of a microbial pathogen, the composition comprising:

35 an amount of a compound according to claim 22 sufficient to inhibit the growth of the microbial pathogen; and

a suitable pharmaceutical carrier.

25. A composition for the treatment of malaria comprising an oxidant agent and a formula XH compound pro-drug.

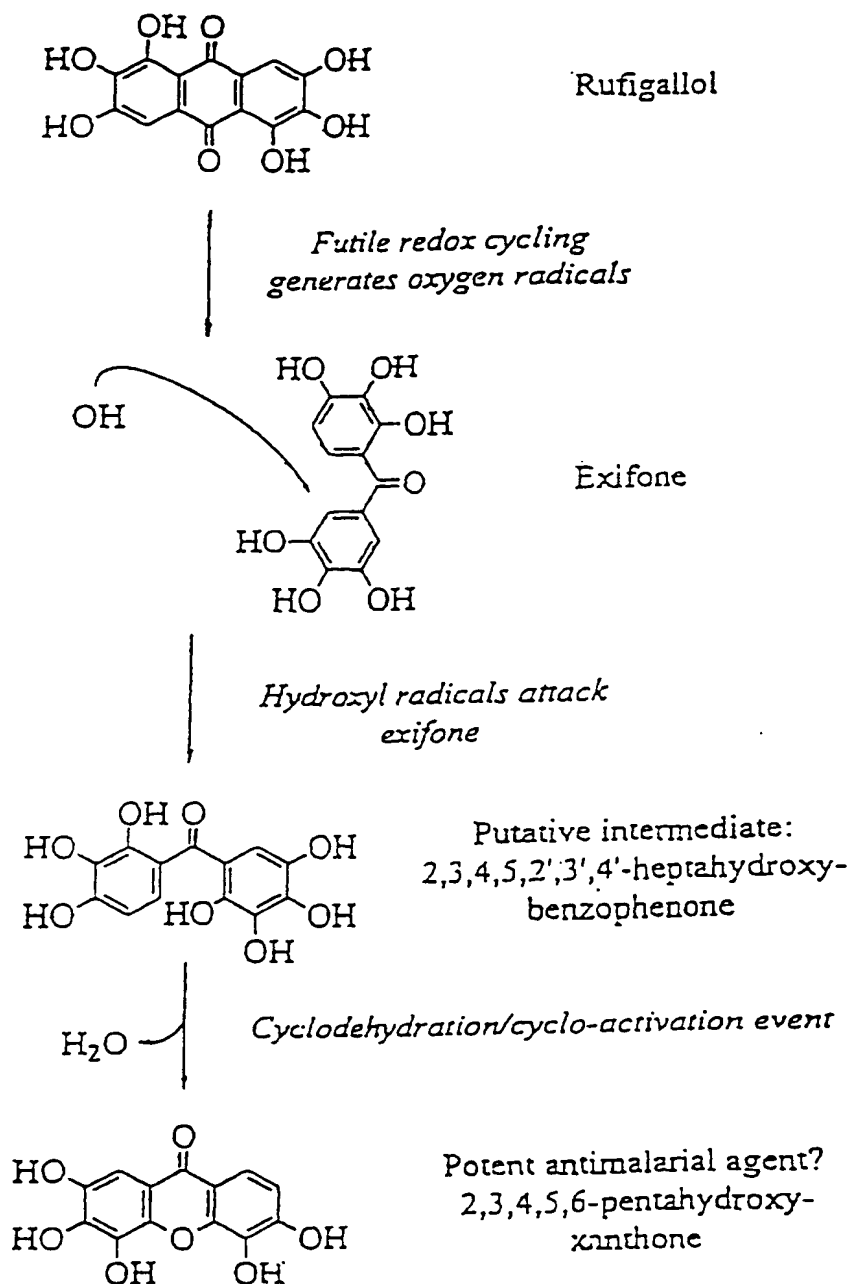
5 26. The composition of claim 25 wherein the oxidant agent is primaquine.

 27. The composition of claim 25 wherein the pro-drug is 3,3'-diethyl-aminoethoxy-2-hydroxy-
10 benzophenone or 2,3,3',4,4',5'-hexahydroxybenzophenone.

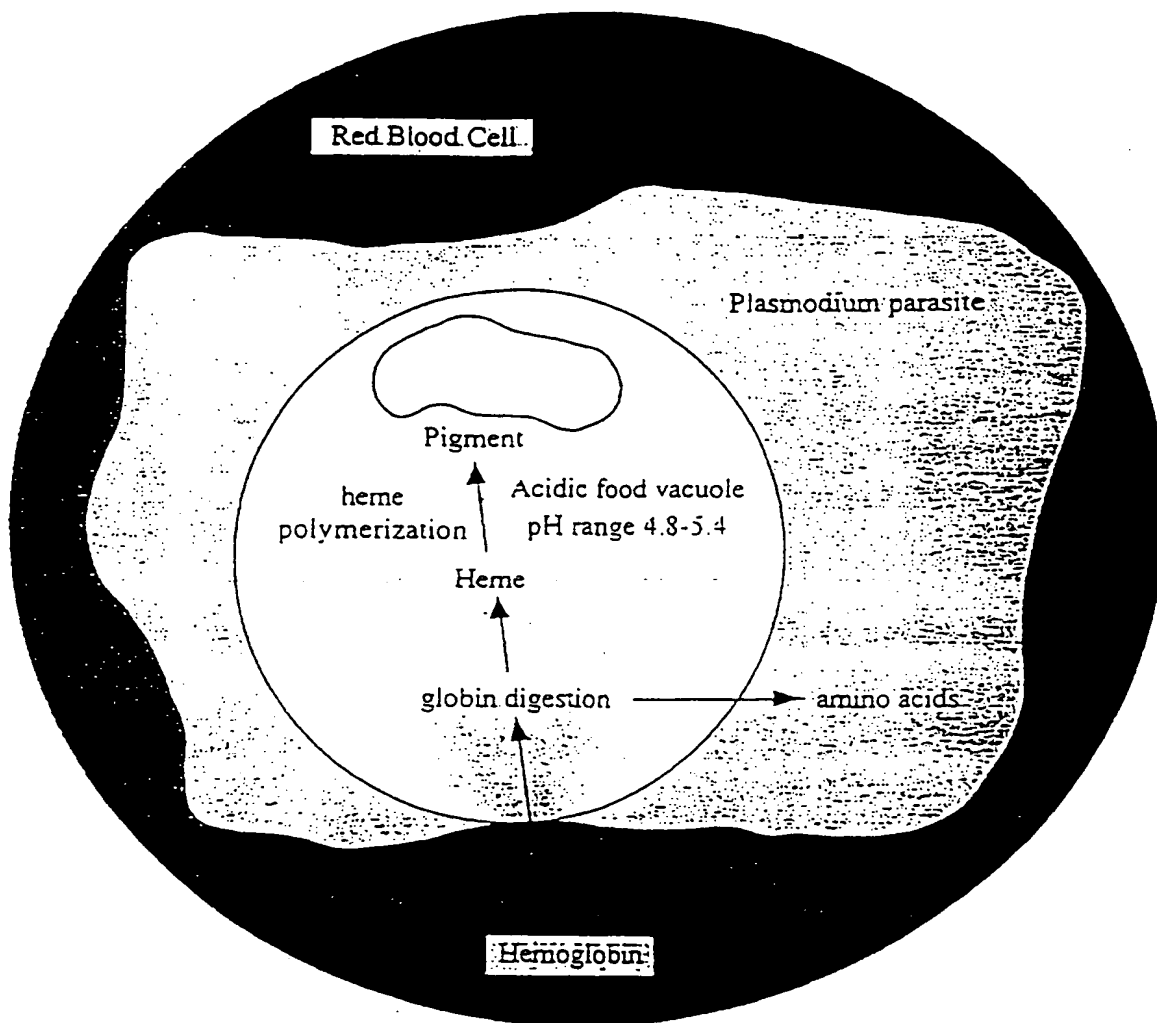
 28. A method of treating malaria comprising administering to a patient a therapeutically effective amount of primaquine in combination with a formula XH
15 compound pro-drug.

 29. The method of claim 28 wherein the pro-drug is 3,3'-diethyl-aminoethoxy-2-hydroxy-benzophenone.

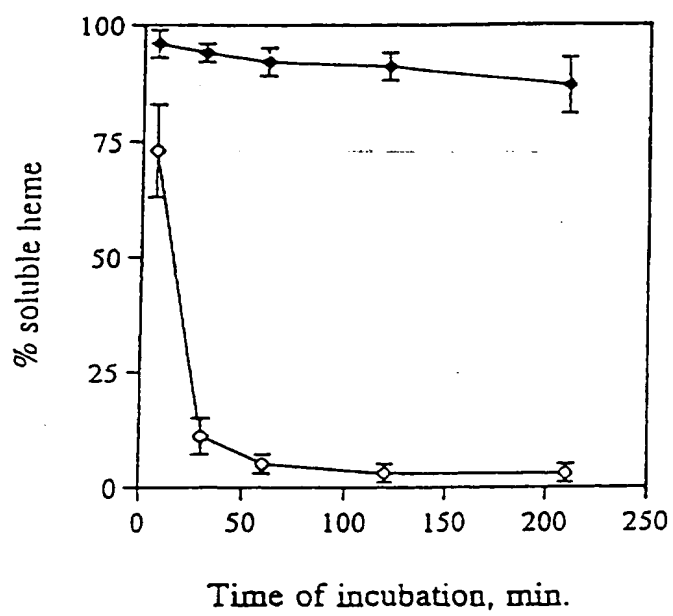
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**FIGURE 1**

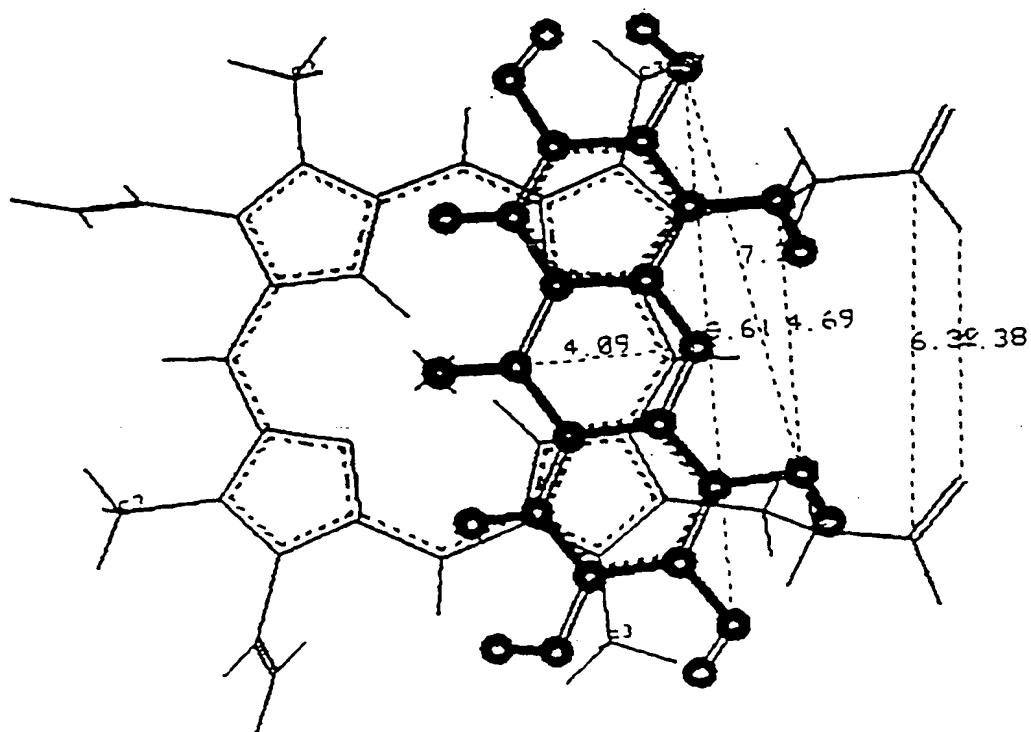
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**FIGURE 2**

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**FIGURE 3**

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**FIGURE 4**

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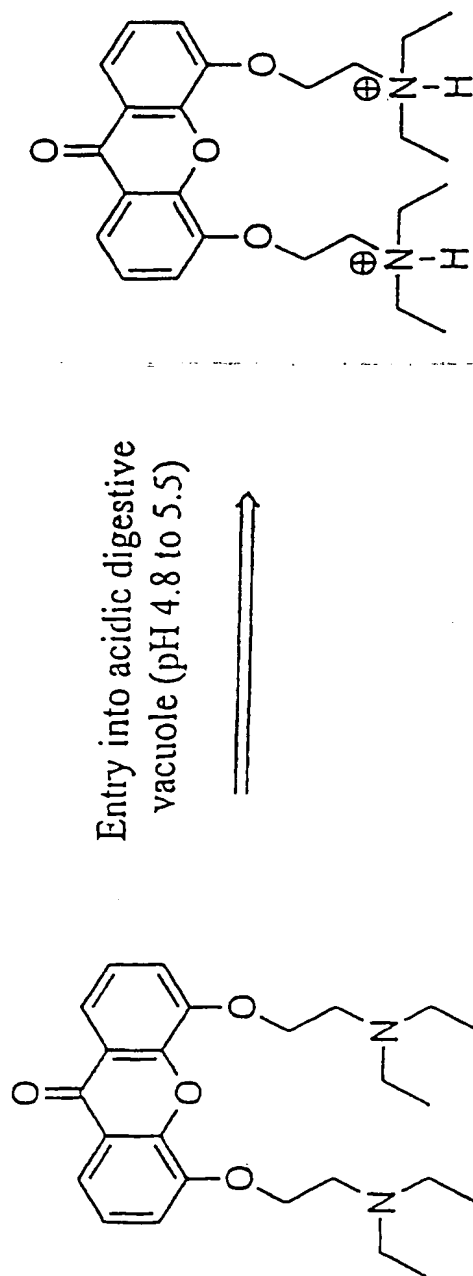


FIGURE 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04965**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A01N 43/00; A61K 31/33; C07D 311/00, 413/00, 413/14

US CL : 514/183, 455; 544/150; 549/383

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/183, 455; 544/150; 549/383

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, REGISTRY, CA, CAOLD, MARPAT

search terms: xanthone, parasit?, fung?, hydroxyxanthone

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HAMBLOCH, H. et al. QSAR with the tuberculostatic activity of polyhydroxy xanthenes and their ¹³ C-NMR chemical shifts. Eur. J. Med. Chem. January 1985, Vol.20, No.1, pages 71-77, especially pages 73-74.	1-2, 4, 6-9, 22-24
X	US 3,887,574 a (ELLIS et al) 03 June 1975 (03/06/75), see entire document, especially column 1-3.	1-2, 4, 6-9, 22-24
X	US 5,281,620 A (DENNY et al) 25 January 1994 (25/01/94), see entire document, especially columns 1-2 and 18.	1-2, 4, 6-9, 22-24

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	Inter document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be of particular relevance	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* A*	document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means		
* P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

29 JUNE 1997

Date of mailing of the international search report

01 AUG 1997

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04965

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-2, 4, 6-9, and 22-24 with species A where A=oxygen

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.